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In re Patent Application of:
Bob BUCHANAN et al.

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For: Ragweed Allergens

Declaration of Oscar L. Frick Pursuant to 37 C.F.R § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Oscar L. Frick, declare as follows:

1. I am an inventor named in the above-referenced patent application.

2. I am a Professor Emeritus of Pediatrics at the University of California at San Francisco.

3. I have reviewed the Del Val et al. abstract (Journal of Allergy and Clinical Immunology (2001) 107 (2): S318) cited by the Examiner, and on which I am an author.

4. My co-inventors and I invented the claimed subject matter prior to February 1, 2001. As evidence of that prior date of reduction to practice of the claimed subject matter, a draft paper prepared well before February 1, 2001 describing our reduction to practice is attached hereto as Exhibit A.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

2/10/06
Date

Oscar L. Frick, MD, PhD
Oscar L. FRICK

A Major New Allergen from Ragweed Unique to Complete Pollen Preparations

Abstract

Ragweed pollen has a lipid layer on the surface which has been extracted and routinely discarded for more than 50 years to produce allergy diagnostic and treatment preparations that are then derived from the defatted grain[1,2] (**Figure 1**). The symptoms in pollen allergy that appear after a few minutes are believed to be due to allergens located on the pollen surface which includes the lipid layer. As has been demonstrated by Marsh et al.[3,4] with defatted ragweed pollen, there are important extracellular allergens released in a short time period --e.g., Amb a 5 in 16 minutes, versus the major allergens described, Amb a 1 and 2, in 12-24 hours. However, neither these authors nor others have found significant differences in the first released allergens from the complete and defatted preparations.

In our work, we show a difference in the population of the first released allergens from complete and defatted pollen. We have identified and characterized an allergen from complete pollen that is very poorly extracted from defatted pollen and is thus missed in current screens. The allergen is obtained from buffer extracts of complete pollen following precipitation with ammonium sulfate and extraction with petroleum ether. The allergen, which appears to be a major pollen glycoprotein, has a molecular mass of 30 kDa and contains at least one disulfide bond. Amino acid sequence data

indicate that the protein has not been previously described from pollen or other sources. After performing IgE-immunoblots with sera of ragweed sensitive patients, we have found that the 30 kDa protein is recognized by all of the 25 individuals tested, thus qualifying it as a major allergen. Similarly a comparative analysis with these sera based on ELISA measurements indicate that the 30 kDa protein is at least as strong an allergen as Amb t 1. Finally, our results are reinforced by the finding that dogs sensitized to commercial preparations of ragweed repeatedly over a long period also recognized the allergen. These findings suggest that the proteins extracted from complete pollen, that contains the 30 kDa allergen and possibly others, should be included in allergy testing and immunotherapy regimes.

Introduction

Pollen extracts for clinical uses have been prepared by convention from defatted pollen grains for more than a half century[5-11]. The protocol that dates from 1922 is based on a simple procedure described by Coca et al.[5] In brief, pollen grains are defatted mechanically in ethyl ether for several hours and the preparation is decanted. The lipid fraction, retrieved in the ether phase, is discarded and the residue containing the pollen grains is dried in warm air. The allergens are then released from the dried pollen grains by incubation in bicarbonate buffer for several hours (**Figure 2a**).

In 1971, Bridger and Protcor[12] demonstrated that pollen grains remain in our nasal mucosa and larynx for only some minutes before being swallowed. During this period, other authors reported the presence of allergens in the ragweed pollen wall. Later, in 1981, Marsh et al.[3,4] demonstrated that several allergens like Amb t 5 are released in a few minutes (16 min), whereas others, such as the major allergen Amb t 1, are released after several hours (12-24 h). This observation suggested that first released proteins are mainly responsible for the early symptoms in pollen allergy. For that reason, we have focused our interest on the first released proteins of pollen. In contrast to the conventional procedure, we attempted to reproduce the release of allergens as occurs in the mucosa. Thus, complete pollen was extracted first with buffer and the proteins obtained were separated from the lipids with petroleum ether (**Figure 2b**). This protocol has allowed us to identify a new group of allergens and characterize one of them, a 30 kDa protein, that has not been previously described. Our data suggest that this protein is a major allergen for humans as well as dogs.

Materials and Methods

Pollen grains. Giant ragweed allergen extract was purchased from Bayer Inc. (Spokane, WA). Complete and defatted Giant ragweed (*Ambrosia trifida*) pollen grains were purchased from Greer laboratories (Lenoir, NC).

Protein quantification. Protein was quantified with the Bradford assay using gamma globulin as standard.[13]

Protein labeling with monobromobimane (mBBr). Protein solutions were reduced with 1 mM dithiothreitol at 100°C, 5 min. The sample was cooled to room temperature and labeled with 0.2 mM mBBr by incubating 20 min at room temperature. The reaction was stopped by adding 10 mM beta-mercaptoethanol and the proteins were precipitated by adding trichloroacetic acid to 12%. After washing with 100% acetone, the pellet was subjected to SDS-PAGE and the extent of protein labeling was visualized by spectroscopy at 365 nm as described by Wong et al.[14]

Glycoprotein staining. After separation by SDS-PAGE, proteins were stained for glycosylation with the in gel GelCode Glycoprotein Staining kit from Pierce (Rockford, IL).

Gel electrophoresis. Samples were reduced by 1 mM dithiothreitol at 100°C, 5 min. and after cooling to room temperature were separated in 10-20% SDS-PAGE.[15] After the run, gels were fixed, stained with Coomassie brilliant blue G-250 and destained in 10% acid acetic. We observed that low molecular weight proteins such as Amb t 5 required reduction by dithiothreitol to be stained effectively with Coomassie blue and that the use of methanol for destaining removes them from the gel.

Immunoblots. Proteins were transferred from 10-20% SDS-PAGE to a nitrocellulose membrane under semi-dry conditions with a 20% methanol solution (25 mM Tris base, 192 mM glycine, 0.1% SDS) for 1 h at 4°C. Nitrocellulose membranes were briefly stained with Ponceau Red to verify the extent of transfer and then blocked by incubating twice with a 3% cow's milk solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.2 % Triton X-100) for 30 min at room temperature. Membranes were then incubated in 1 to 10 dilution of sera in the same solution overnight at 4°C. Finally,

membranes were incubated at room temperature for 1 h in a 1000X dilution of secondary anti-human IgE conjugated to horseradish peroxidase (Sigma) and reactive protein identified with 3,3',5,5'-tetramethylbenzidine (TMB) substrate kit for peroxidase from Vector laboratories (Burlingame, CA).

Skin tests. Procedures to measure the type I hypersensitivity reaction by skin tests with sensitized dogs have been described elsewhere[16]. In brief, 0.5% Evans blue dye (0.2 ml/kg) was injected intravenously 5 minutes prior to skin testing. Aliquots of 0.1 ml of the test protein solution in half log dilutions were injected intradermally on ventral abdominal skin. Skin tests were read by the same experienced blinded observer scoring the two perpendicular diameters of each blue spot. Appropriate negative controls (diluted in PBS) were included for each animal tested.

Calculation of Relative allergenicity. Equal amount of protein, either purified or in pollen extracts, were injected and assigned a relative value indicating the minimal quantity producing a wheal: 330 ng protein = 1, 100 ng = 2, 33 ng = 3, 10 ng = 4, 3.3 ng = 5, 1 ng = 6 and 0.33 ng = 7. We then summed the values for each purified protein or extract for the two groups of dogs tested [4 old (7-year-old) and 5 young (2-year-old) dogs.

Protein extraction and allergen purification. An adaptation of the method of Marsh et al. 1981 was followed[3, 4]. To work out the procedure, an extract was prepared from 10 g of pollen (complete or defatted) and subjected to different exploration treatments. For the purification of proteins, 100 g of complete pollen was used. In brief, the pollen was suspended at 1 g to 10 ml of cold buffer [50 mM Tris-HCl pH 7.4 containing 1 µM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA-Na] and

stirred gently for 30 min at room temperature. The mixture was centrifuged for 10 min, 25,900 x g, 4°C. The pellet, containing the pollen grains, was set aside and the supernatant fraction was recentrifuged and filtered through Whatman quantitative filters. Ammonium sulfate was added to 95% saturation yielding a floating pellet that was recovered by centrifugation (10 min, 25,900 x g, 4°C) and resuspended to a minimal volume with 20 mM Tris-HCl pH 7.5, containing 200 mM NaCl. With both complete and defatted pollen, the high quantity of lipids was removed by extraction with an equal volume of petroleum ether. The mixture was centrifuged (10 min, 48,400 x g, 4°C) and the organic fraction was discarded. The petroleum ether step was repeated at least 4 times. The resulting clarified aqueous solution was filtered through a 0.2 µM filter and, in the case of 100g pollen, separated on a Sephadex G-50F gel filtration column (2.1 x 90 cm) equilibrated and eluted with the same buffer used to dissolve the sample. The fractions were analyzed by 10-20% SDS-PAGE, combined according to protein size and dialyzed against 10 mM K-phosphate buffer pH 7.0 overnight at 4°C. The remainder of the procedure is described for 100g pollen as starting material.

30 kDa protein. The combined Sephadex G-50F fractions of the proteins from complete pollen were dialyzed against 10 mM K-phosphate pH 7.0 and applied first to a 6 ml Resource S column and then to a 6 ml Resource Q column, both equilibrated with 20 mM K-phosphate pH 7.0. The 30 kDa protein was not retained in either case and was recovered in the column pass-through fractions. A considerable amount of contaminants was retained on the columns and thus removed from the 30 kDa protein. The fractions containing the 30 kDa protein were subjected to precipitation with ammonium sulfate, 95% saturation, and centrifuged for 10 min, 48,400

x g, 4°C. The supernatant fraction was discarded and the pellet was resuspended in 2-3 ml volume of 50 mM K-phosphate, pH 7.0, containing 2.0 M ammonium sulfate. The fraction was applied to a 1 ml Resource Isopropyl column equilibrated with the same buffer. The 30 kDa protein was eluted at 1.7 M using a 60 ml gradient ranging from 2 to 0 M ammonium sulfate. The fractions containing the 30 kDa protein were localized by SDS-PAGE (using mBBr labeling and Coomassie blue staining), combined and dialyzed against 5 mM K-phosphate, pH 7.0. Sodium-acetate pH 4.75, was then added to 30 mM and the sample was applied to a 6 ml Resource S equilibrated with the same buffer. The 30 kDa protein was eluted at 100-200 mM NaCl in a 120 ml gradient ranging from 0 to 300 mM NaCl. The fractions were neutralized by adding 50 mM K-phosphate pH 7.0, dialyzed against 10 mM of the same buffer, concentrated by ultrafiltration with a YM-10 Amicon membrane and stored at –70°C. Protein was quantified using the Bradford assay.

30 kDa protein (alternate procedure). Following addition of 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 M NaCl, the combined Sephadex G-50F fractions were applied to a 18 ml Concanavalin A affinity column (Sigma Chemical Co., St. Louis, MO) equilibrated with the same buffer. The 30 kDa protein was retained and eluted with a solution of 20 mM Tris-HCl pH 7.5, 0.5 M NaCl and 0.5 M methyl-alpha-D-glucopyranoside. The fractions containing the 30 kDa protein were combined and dialyzed against 5 mM K-phosphate buffer pH 7.0 using a membrane with a 25,000 M.W. cutoff pore. Finally, the protein was applied to a 6 ml Resource S column equilibrated with 20 mM Na-Acetate, pH 6.0, and was recovered in the pass-through fractions.

Amb t 5. Ammonium sulfate was added to 2.6 M to the low molecular weight Sephadex G-50F fractions from complete pollen containing Amb t 5. The resulting solution was fractionated on a 1 ml HiTrap Phenyl Sepharose column equilibrated with 200 mM phosphate buffer, pH 7.0, and eluted with a 50 ml of ammonium sulfate gradient ranging from 2.5 to 0 M in this same buffer. Pure Amb t 5 was recovered in a single peak at approximately 0.8 M ammonium sulfate, dialyzed against 5 mM K-phosphate buffer, pH 7.0, and stored at -70°C for further experiments. Protein was quantified using a molar coefficient of extinction at 278 nm of 5800[22].

Amb t 3 and cytochrome c. The maximal yield of Amb t 3 was obtained from complete pollen and cytochrome c from defatted pollen. The Sephadex G-50F fractions containing proteins of about 10-20 kDa from the respective pollen preparations were combined and applied to a 6 ml Resource S column equilibrated with 20 mM K-phosphate buffer, pH 7.0. Amb t 3 and cytochrome c were separated with a 120 ml gradient from 0 to 500 mM NaCl in 20 mM K-phosphate buffer pH 7.0. Amb t 3 was eluted at 100-120 mM and cytochrome c at 150-170 mM NaCl. The presence of Amb t 3 was confirmed by adding a crystal of potassium ferricyanide to the fractions to oxidize the copper of Amb t 3, thereby turning the solution blue. Fractions containing Amb t 3 were combined and made 2 M with ammonium sulfate. The final purification of Amb t 3 and cytochrome c was achieved by separation through a 1 ml HiTrap Phenyl Sepharose column equilibrated with 200 mM K-phosphate buffer, pH 7.0. The column was eluted with a 60 ml ammonium sulfate gradient ranging from (1) 1.75 to 0 M for Amb t 3, which was eluted at 1.4 M, and (2) 2.0 to 0 M for cytochrome c, eluted at 1.2 M. The purified proteins were then dialyzed against 10 mM K-

phosphate buffer, pH 7.0, and stored in aliquots at -70°C. Protein content was quantified using the Bradford assay and, in the case of Amb t 3, using a molar coefficient of extinction at 278 nm of 26600[22].

Amb t 1and 2. The largest quantity of Amb t 1-2 was obtained from defatted pollen. The Sephadex G-50F fractions containing proteins of 35 kDa and greater were dialyzed against 20 mM Tris-HCl, pH 7.9, containing 14 mM beta-mercaptoethanol. The following steps were modified from the procedure of King's group.[17-21] The protein solution was applied to a 6 ml Resource Q column equilibrated with 20 mM Tris-HCl, pH 7.9, and eluted with a gradient of 240 ml ranging from 0 to 500 mM NaCl. As described earlier by King's group, the proteins were eluted at about 50 mM NaCl. Ammonium sulfate was added to 2.5 M and the solution was applied to a 1 ml Resource Isopropyl column equilibrated with 100 mM K-phosphate, pH 7.0, containing 2.5 M ammonium sulfate. Amb t 1 and Amb t 2 were eluted at about 1.4 M ammonium sulfate in a 100 ml gradient ranging from 2.5 M to 0 M. The positive fractions were identified as above, combined, concentrated by ultrafiltration through a YM-30 Amicon membrane, dialyzed against 10 mM K-phosphate pH 7.0 and stored in aliquots at -70°C. The positive fractions were shown to have the correct molecular mass by SDS-PAGE; allergenicity was confirmed by skin tests with ragweed-sensitive dogs. Protein was quantified with the Bradford assay.

Results

The project began with the testing of complete and defatted giant ragweed pollen extracts with our atopic dogs[16] that had been sensitized with a defatted commercial pollen extract. The objectives were (1) to determine whether the two extracts, complete and defatted, contained different allergens, and (2) to compare the major allergen Amb t 1 (40 kDa) to the purified first released allergens, Amb t 5 (5 kDa), Amb t 3 (10 kDa) and cytochrome c (12 kDa).

We made the comparison with skin tests by injecting identical quantities of each purified allergen or allergen fraction (first released proteins of complete or defatted pollen). We assessed relative allergenicity by determining the minimal quantity of each purified allergen or extract fraction required to elicit a wheal (**Figure 3**). We observed differences between the young and old dogs. In the case of the old dogs, the strongest reaction was given by the major allergen, Amb t 1,[1,17-21] followed closely by Amb t 3 and Amb t 5. In this case, the complete and defatted pollens gave a similar response. By contrast, with the young dogs, Amb t 1 was by far the strongest allergen followed by cytochrome c. The response to Amb t 3 and Amb t 5 was quite low and the allergenicity of the defatted pollen was stronger than for the complete counterpart. The results suggest that the commercial extract, which was prepared with defatted pollen, contained low levels of the first released proteins, thus requiring additional time for sensitization and eliciting a response only in the old dogs studied. This conclusion was confirmed by immunoblots with human sera (see below).

This result prompted us to carry out a more complete analysis of the allergens present in the first released proteins of complete and defatted ragweed pollen. Owing to the large amount of lipids recovered in aqueous extracts of complete pollen grain, we developed the procedure described below (**Figure 2b**): First, we resuspended the complete or defatted pollen grains (0.1 g/ml) in 50 mM Tris-HCl, pH 7.5, and stirred gently for 30 min. As the next step was a gel filtration, the preparation had to be concentrated and largely freed of lipids and pigments. To that end, we found that 95% ammonium sulfate coprecipitated the lipids and the proteins and resulted in a floating precipitate that was easily isolated (**Figure 4**).

We collected the precipitate from the complete and defatted extracts by centrifugation, redissolved and then extracted it at least five times with petroleum ether. The clarified redissolved pellet solution was fractionated using a Sephadex G-50F gel filtration column. The fractions from the two types of pollen were then examined with respect to:

a) Total protein using Coomassie blue stain (**Figure 5a**),

b) Proteins containing sulfhydryl groups using a fluorescent probe, monobromobimane, applied after reduction with dithiothreitol (**Figure 5b**),[14]

c) Allergens using a mixture of sera from 10 patients positive to giant ragweed (**Figure 5c**).

Significant differences were noted between the complete and defatted pollens. Standing out was a 30 kDa protein (identified with a wedge) that contained a sulfhydryl group and was delayed in the gel filtration column,

so that it was recovered with the low molecular weight proteins, e.g., Amb t 5 (**Figures 5a and 5b**). The 30 kDa protein appeared to be recognized by the mixture of human sera (**Figure 5c**). By exposing the gel filtration fractions to individual sera, we found that the 30 kDa protein was recognized by sera of all patients tested, whereas the other allergens were not (data not shown). This finding indicated that the 30 kDa was a major allergen (see **Figures 7a** and **7b** below). In addition, several other described proteins not previously described, were found to bind human IgE. These include (1) an 8-10 kDa disulfide protein (G-50F fraction #36) in complete pollen extract, just above Amb t 3 (Amb t 3 is identified with a star in **Figure 5c**), and (2) a 30 kDa protein in the defatted extract that is not delayed by the gel filtration (G-50F fraction #16, identified with a diamond in **Figure 5c**). Finally, as Marsh and collarborators[3,4] reported, we found the level of the major allergen Amb t 1-2 to be quite low in the first released proteins from the defatted preparation. A significantly higher quantity of this allergen as well as Amb t 3 and Amb t 5 was, however, found in the complete pollen extract (data not shown).

Because of its apparent allergenic properties, we purified the 30 kDa protein from complete pollen to homogenicity. In characterizing the protein, we found it to have properties of known allergens[23-26]—i.e., it (1) is a glycoprotein (**Figure 6a**), (2) has at least one disulfide bond (**Figure 6b**) and (3) has a pI about 8.0 (data not shown). As in the case of many allergens, glycosylation and the presence of a disulfide bond are known to confer stability and resistance to proteolysis. The finding of a glycan moiety in the protein led us to find another interesting property. After the gel

filtration separation, the 30 kDa protein was strongly retained (>90%) on a glycoprotein affinity column, concanavalin A. This feature simplifies the purification of the protein to a few steps (see Material and Methods). Further experiments indicated that the 30 kDa protein is also retained at a lower level by lectin affinity columns (data not shown). The affinity data suggest that the glycan moiety is composed mainly of alpha-D-mannose and alpha-D-glucose.

The next question was to determine whether the 30 kDa is a known protein. We therefore obtained partial amino acid sequences by mass spectrometry (analysis carried out at the University of California, Davis). The results indicate that aside from marginal similarity to envelop glycoproteins, the 30 kDa protein has not been previously described from pollen or other sources.

Amino acid sequence of tryptic peptidese

1. L/I L/I SGISNTVYANPK
2. PTSFN L/I ATK
3. L/I YGLVQFNR
4. FY L/I FSTK
5. FYATEV L/I D L/I D*

* Homology with envelope glycoproteins

The next query was to assess the allergenic importance of the 30 kDa protein with ragweed-sensitive patients. An allergen is qualified as being major if recognized immunologically by at least 50% of a minimum of 15

sensitive patients[24] In our case, we selected 35 persons who showed sensitivity to giant ragweed pollen by experiencing allergic rhinitis or a positive percutaneous skin test (> 3 mm induration). Among these reactors, we identified 11 persons positive to the Pharmacia ImmunoCAP assay (RAST). We determined the allergic response of all 35 patients with IgE-immunoblots using our first released protein extract of complete pollen containing the 30 kDa protein. Among these patients, 15 were strongly positive to the complete pollen extract (13 to a commercial ragweed preparation), whereas, as noted above, only 11 were positive to the Pharmacia ImmunoCAP assay. However, a total of 26 patients (including all of the 15 positives to the complete pollen extract) reacted to the purified 30 kDa protein (**Figure 7a**), thus qualifying it as a major allergen. Nine of the 35 patients appeared not to react to the ragweed preparations tested with the immunoblot procedure. At least two patients (nos. 4 and 7) who were negative when tested with the commercial extract were positive with the first released proteins of complete pollen as well as with the 30 kDa allergen (**Figure 7b**). It is not known whether the high reactivity seen with the 30 kDa is due to its potent allergenicity or to cross-reactivity with patients sensitive to other pollens.

Briefly, of the 35 patients tested we found:

- 11 positive to the Pharmacia ImmunoCAP assay,
- 15 positive to first released proteins of complete pollen extract, and
- 26 positive to the purified 30 kDa protein.

To summarize, the sera data indicate that the 30 kDa protein is a major allergen and, furthermore, that use of commercial defatted extracts may give false negatives. In our study, we identified patients who were positive to the complete pollen extract, but were negative to the ImmunoCAp assay. Thus, there were at least 4 strong reactors to complete pollen extract (patient nos. 7, 9 18 and 26) who failed to react with the ImmunoCap screen (all data not shown). Based on this finding, the ImmunoCap assay misses about 25% of ragweed-sensitive patients (4 of the 15 patients who responded to the complete pollen extract). In addition, we identified 15 reactors to the 30 kDa protein (patients nos. 4, 7, 8, 9, 12, 17, 18, 19, 22, 23, 24, 26, 31 and 34) who were not detected with the other tests. It is not known whether these patients were sensitive to other pollens.

A comparative assessment of allergenicity using an ELISA protocal confirmed the immunoblot results in identifying the 30 kDa protein as allergen (**Figure 8**). Moreover, the 30 kDa protein bound human IgE from 10 ragweed-sensitive patients more strongly than any of the known allergens tested. The results provide further evidence that the 30 kDa protein is a major allergen in ragweed pollen.

A remaining question was whether the allergenicity of the 30 kDa protein could be detected *in vivo*. To this end, we tested the purified protein with an atopic dog colony sensitized to giant ragweed pollen and observed a hypersensitive response. We obtained a positive response with 16 of the 19 animals tested (**Table 1**). As expected from the results in **Figure 3**, the old dogs were more sensitive to the 30 kDa protein (by 10-fold) than their young counterparts. In addition, whereas the old dogs were uniformly

sensitive to the new allergen, 20% of the young dogs were not. These results indicate that the low level of 30 kDa protein present in commercial preparations is sufficient to sensitize dogs if injected repeatedly over an extended period. This observation also supports the conclusion that the defatted commercial extract is deficient in major allergens.

Conclusions

• We have developed a procedure to extract allergens associated with the extracellular lipid layer of pollen. The results suggest that the procedure can be used to generate improved formulas for allergy diagnostics as well as immunotherapy regimes.

• We have isolated a new group of allergens that are released form complete or defatted ragweed pollen within minutes. Included is a previously undescribed 30 kDa protein that seems to be a major allergen. This protein is associated with the lipid layer and is discarded with the commercial defatting process. The protein (1) has a molecular mass of 30 kDa, (2) is glycosylated and (3) has at least one disulfide bond.

• As a major allergen, the 30 kDa protein may be useful in allergy assays and immunotherapy regimes for both humans and animals.

• In addition, we have identified a previously unreported 8-10 kDa protein with at least one disulfide bond and a second protein of 30 kDa that both bind IgE.

• The 30 kDa protein may be an allergen common to other pollens. Further experiments will determine if the protein has homologous forms in pollens such as birch and rye grass.

• The large quantity of disulfide proteins released in the first minutes from complete pollen grains are possible targets of the NADP/thioredoxin system. As demonstrated earlier with wheat, milk, and more recently with pollen.[27-29] reduction by thioredoxin renders certain allergens hypoallergenic and hyperdigestible. Thus the application of the thioredoxin system by a nasal spray could be effective in disarming first released allergens.

• We propose to apply the proteins reduced by the NADP/thioredoxin to develop safer and efficient immunotherapy regimes.

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soluble fraction of ragweed pollen. *Journal of Allergy and Clinical Immunology* 105 (1): 980.

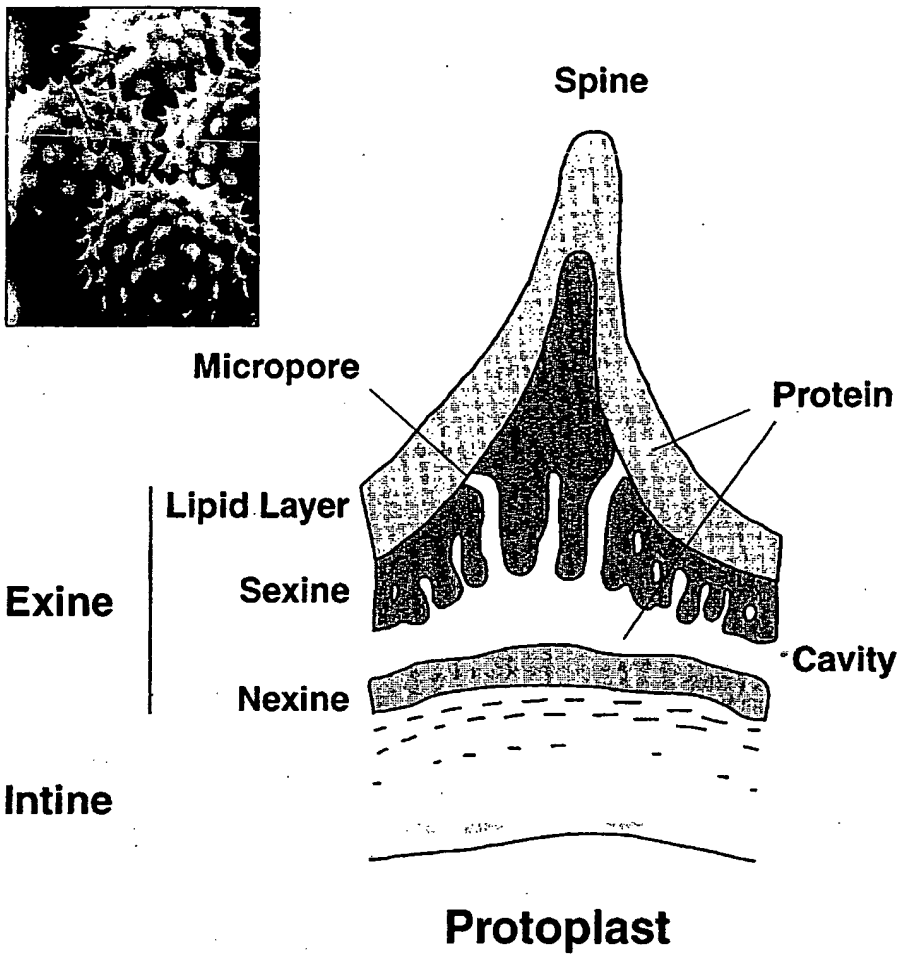

Figure 1. Structure of the wall of pollen ragweed. Howlett et al. 1973, J. Cell. Sci.

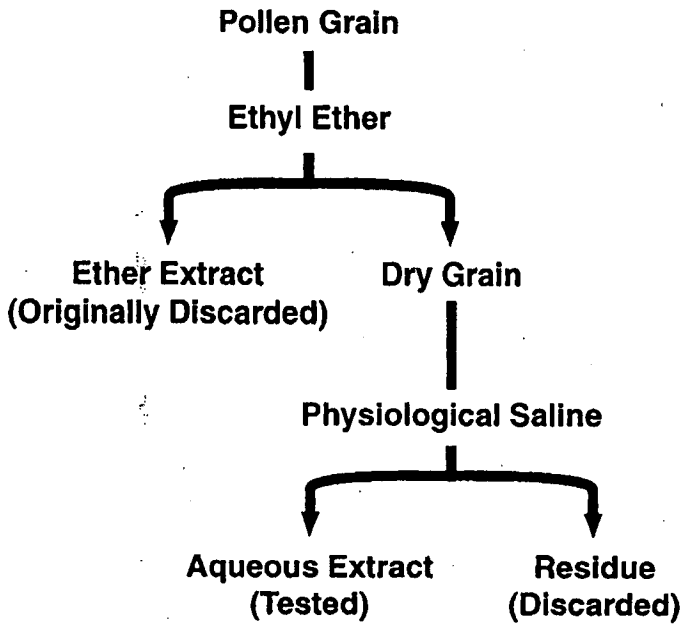

Figure 2a. Contemporary procedure for producing clinical pollen preparations (testing and desensitization).

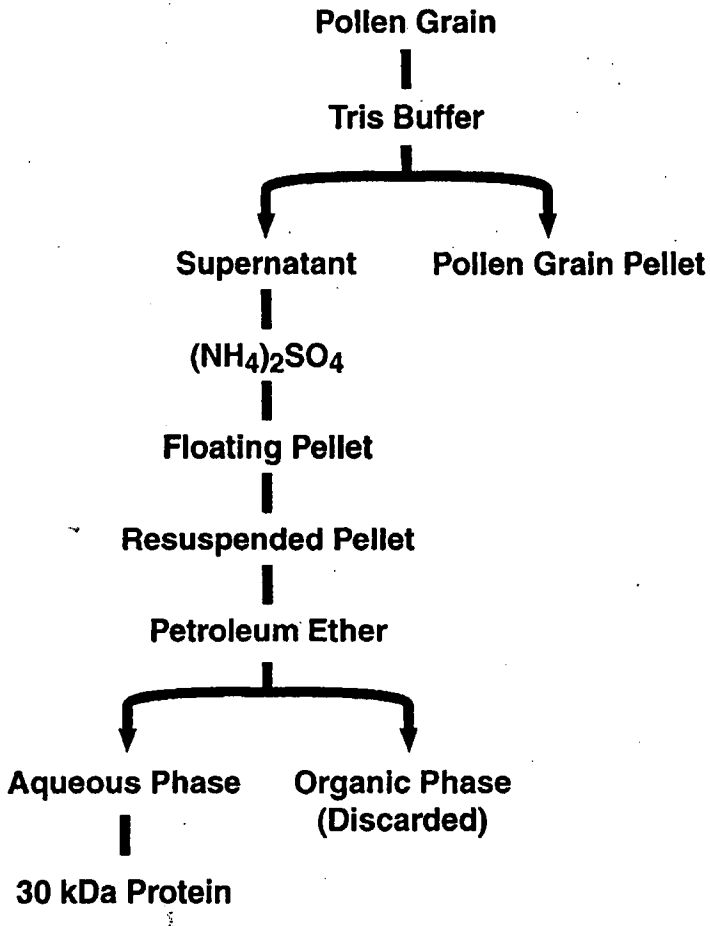

Figure 2 b. Procedure for the extracting 30 kDa protein from ragweed pollen

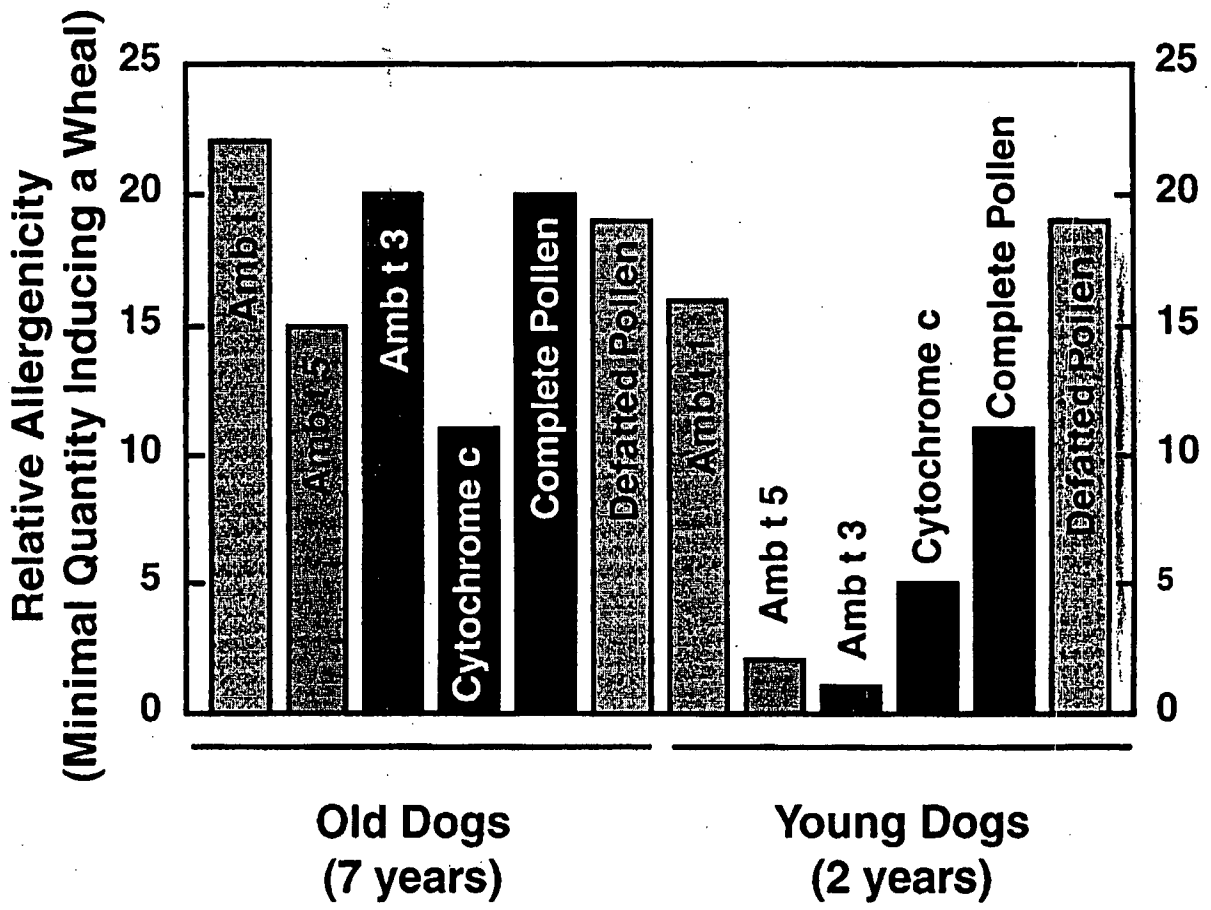

Figure 3. Relative response to allergens purified from giant ragweed.

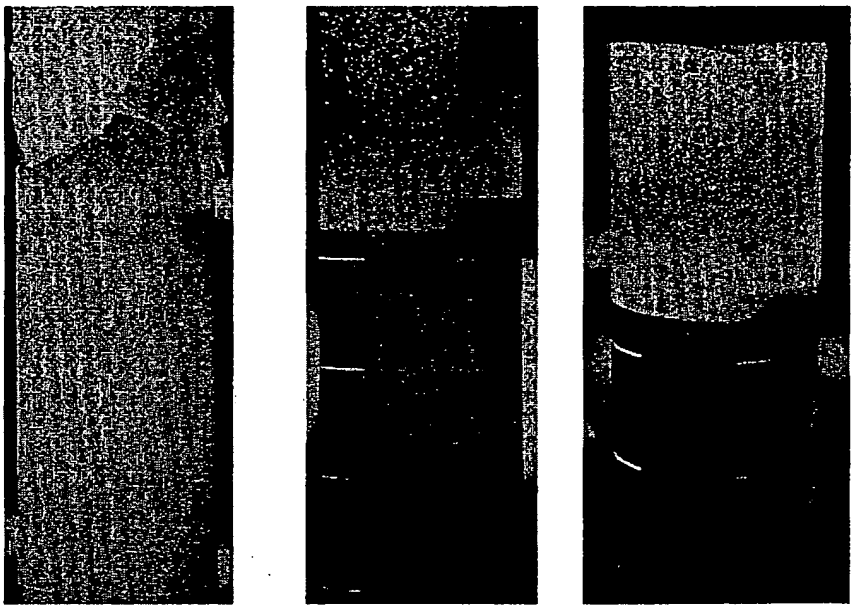

Figure 4. Coprecipitation of lipids and first released proteins in 95% ammonium sulfate "protein soap".

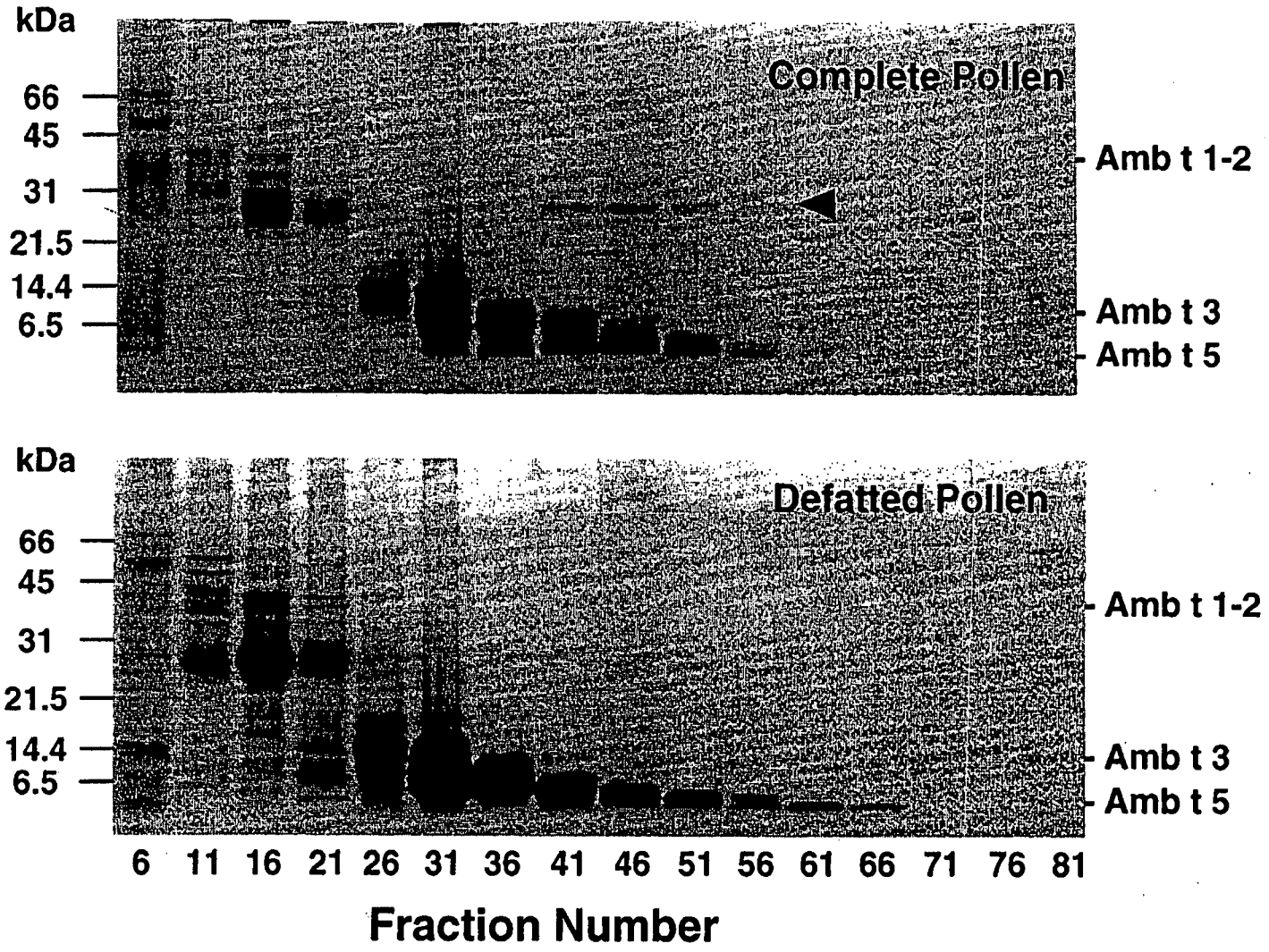

Figure 5a. Protein staining/Coomassie blue. Profile of protein from complete and defatted ragweed pollen on a Sephadex G50F column. Proteins were analyzed by SDS-PAGE and gels were stained with Coomassie blue.

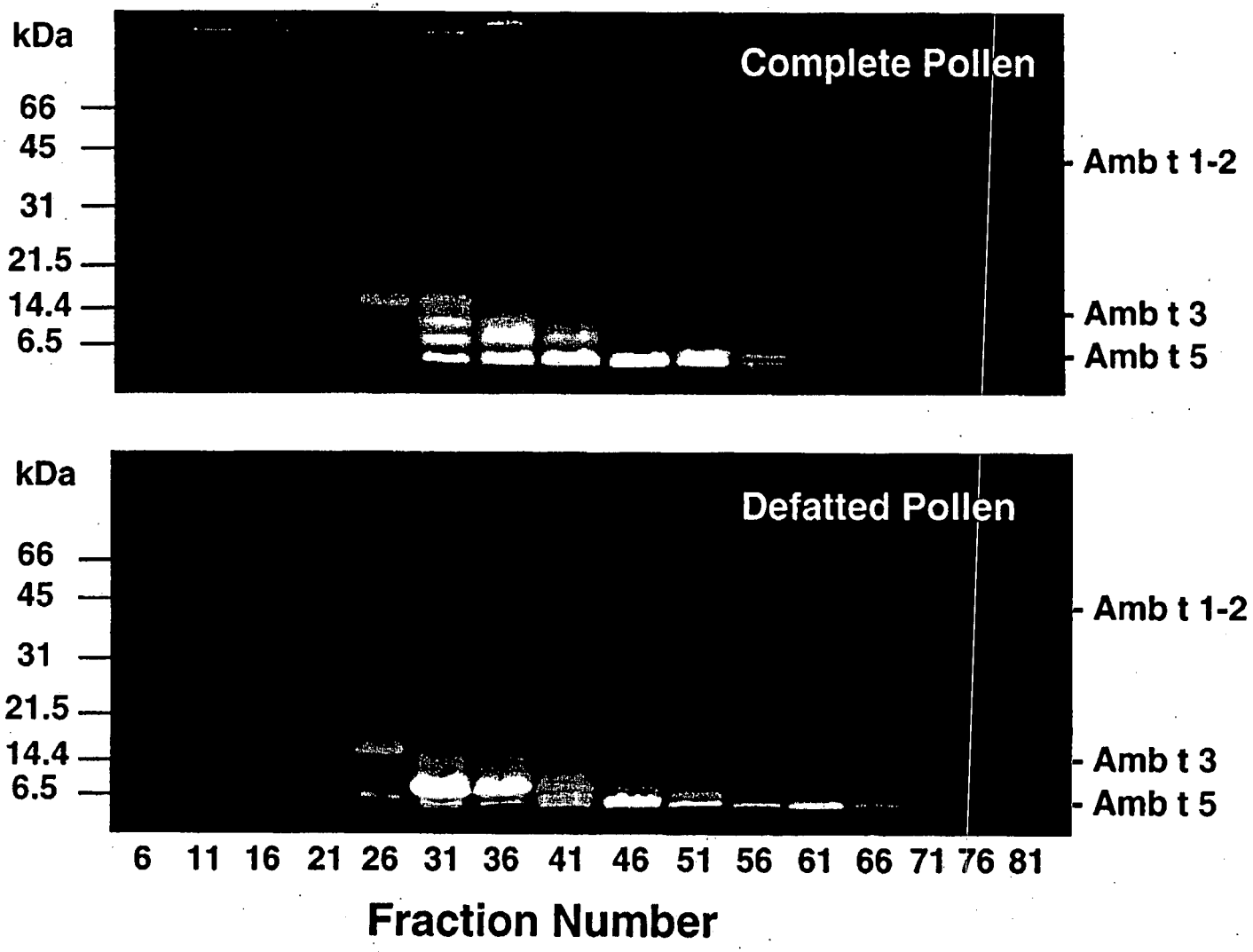

Figure 5b. Sulfhydryl determination/monobromobimane. Profile of protein from complete and defatted ragweed pollen on a Sephadex G50F column. Sulfhydryl groups of proteins were labeled with monobromobimane and analyzed by SDS-PAGE (UV light).

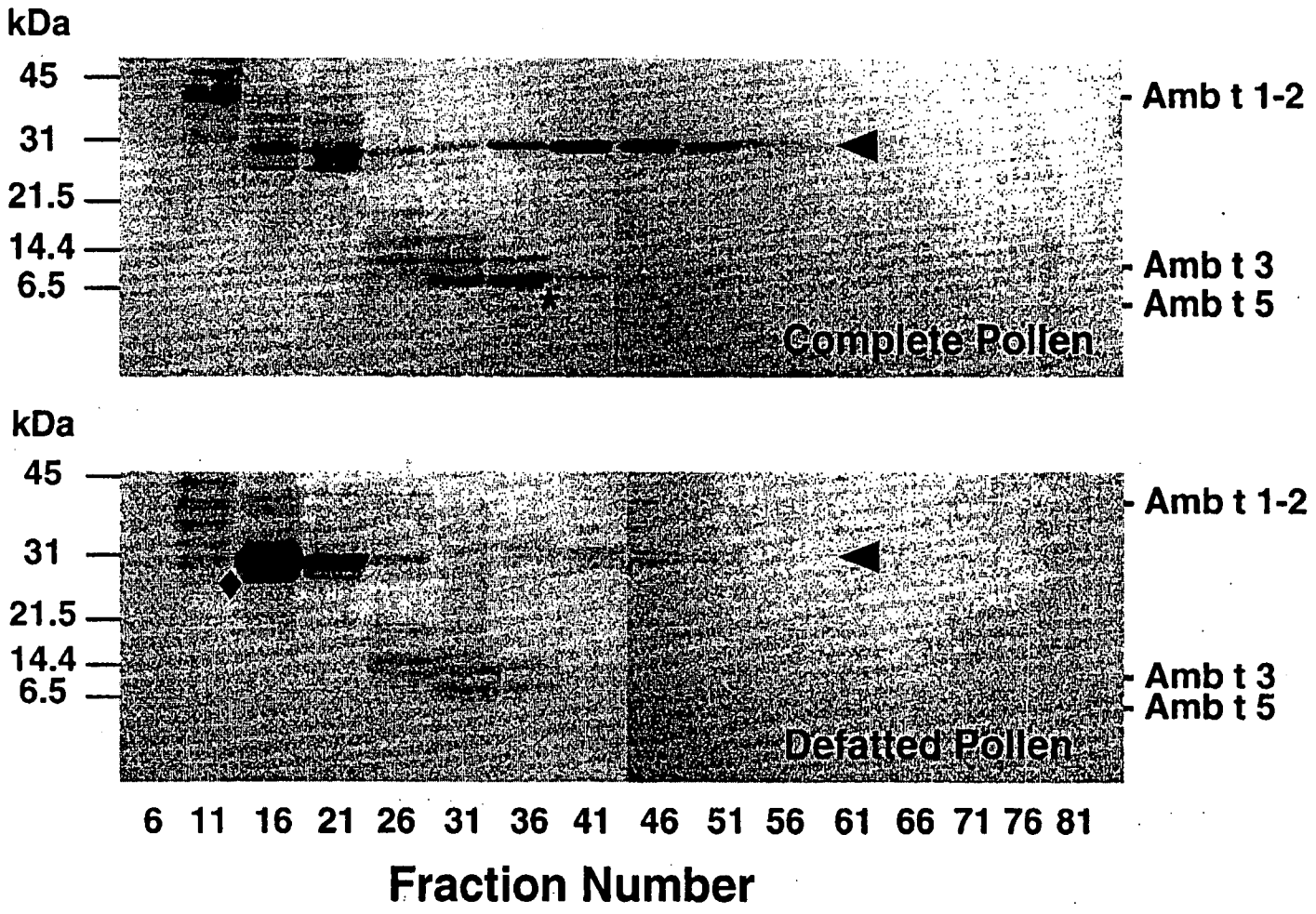

Figure 5c. Allergen determination/IgE Immunoblot. Profile of protein from complete and defatted ragweed pollen on a Sephadex G50F column. Proteins were separated by SDS-PAGE transfered to nitrocellulose and probed with IgE of combined sera from ragweed patients. Complete pollen: ◄ 30 kDa protein and * 8-10 kDa protein. Defatted pollen: ♦ 30 kDa protein.

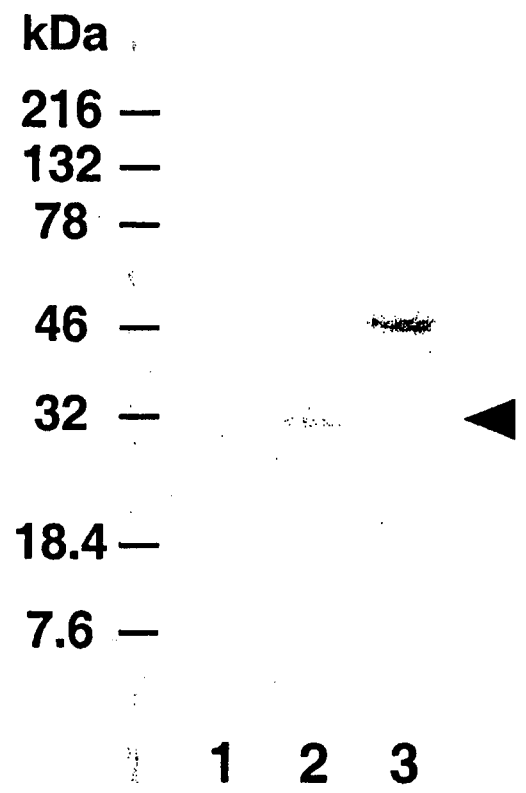

Figure 6a. Demonstration that the purified 30 kDa protein from complete ragweed pollen is glycosylated. SDS-PAGE (10-20%). 1. Soybean trypsin Inhibitor (negative control) 2. 30 kDa protein and 3. Horseradish peroxidase (positive control).

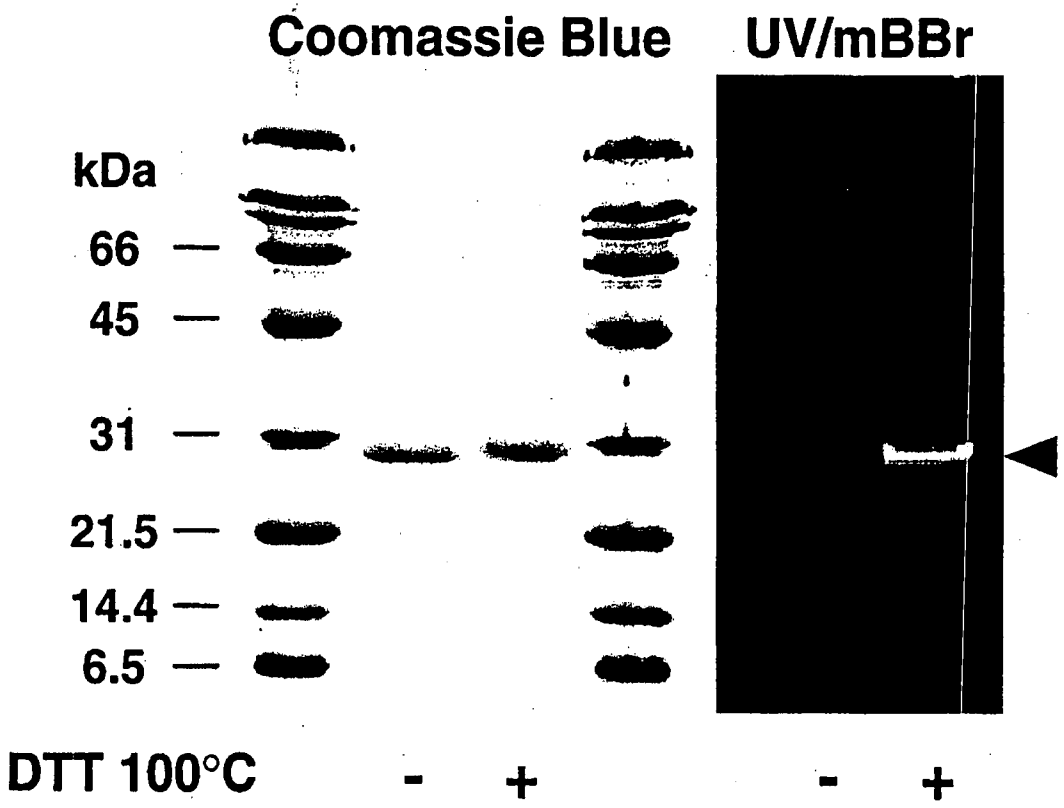

Figure 6b. Demonstration that the purified 30 kDa protein from complete ragweed pollen contains at least one disulfide bond. SDS-PAGE (10-20%). Coomassie blue and UV/mono-bromobimane (mBBr).

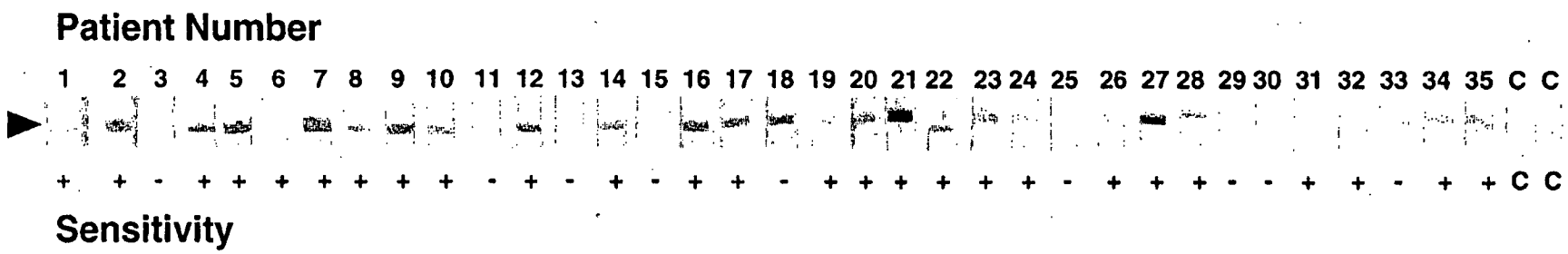

Figure 7a. IgE response of sera from 35 patients to the purified 30 kDa protein of complete ragweed pollen. SDS/PAGE immunobloting.

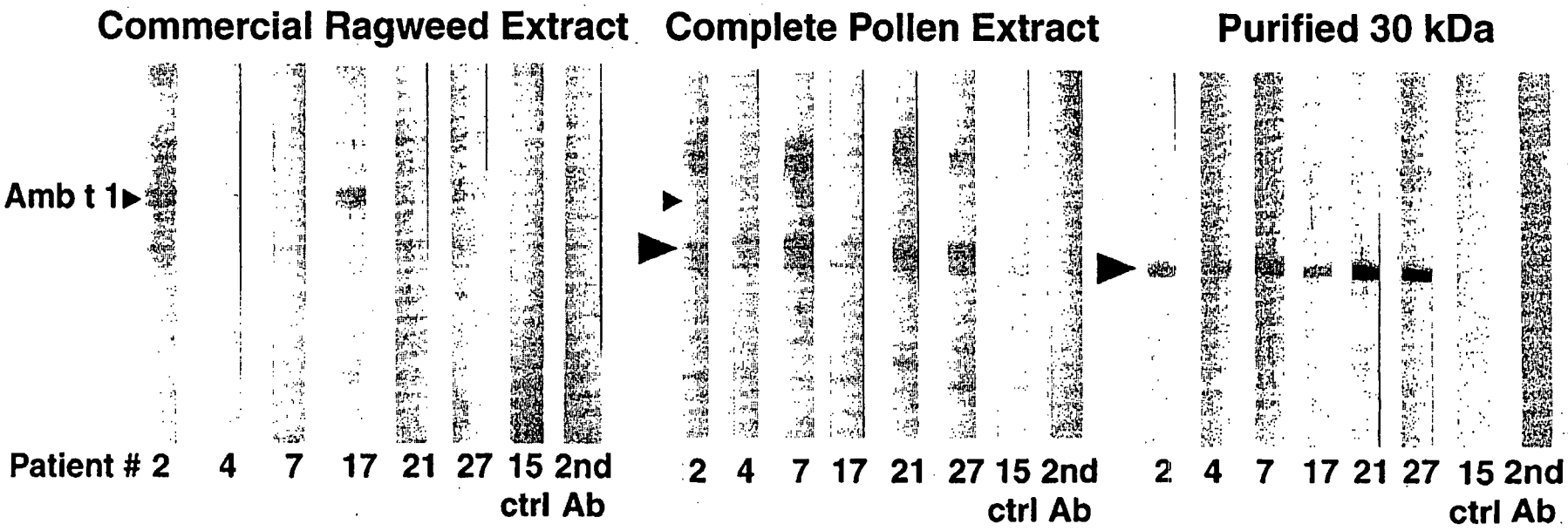

Figure 7b. IgE response of sera from selected positive patients to commercial and complete crude extracts, and to the purified 30 kDa protein from complete ragweed pollen. SDS/PAGE immunoblotting.

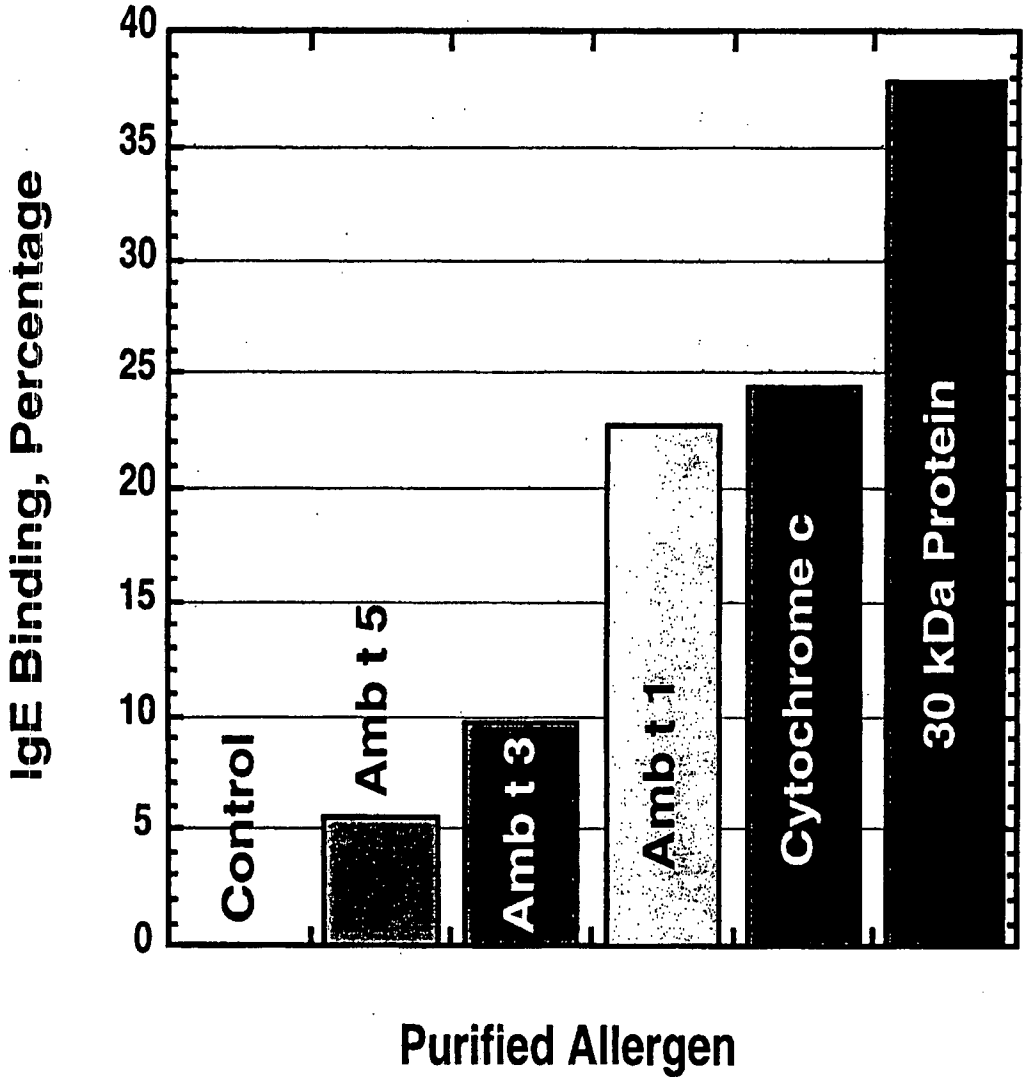

Figure 8. Percentage of human IgE binding to major allergen of ragweed pollen. Determined by ELISA with sera from 10 ragweed-sensitive patients.

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